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ANTIOXIDANT, ANTIARTHRITIC, AND ANTIMICROBIAL ACTIVITY OF SYNTHESIZED SILVER NANOPARTICLES USING *ANNONA SQUAMOSA* ROOT UNDER IN-VITRO STUDY

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ABSTRACT

Biosynthesized silver nanoparticles from ethanolic extract of root of *Annona squamosa* shows the strong antioxidant and antiarthritic activities. The characterization of synthesized silver nanoparticles shows the shape, size, particle range by FTIR, UV-Visible, SEM, XRD and EDAX analysis. SEM analysis shows the morphology of silver Nanoparticle. The silver nanoparticles lie between 74.08 to 134.2nm region and the average size of silver nanoparticles is 455.9nm which shows the shape of silver nanoparticles as spherical. The antioxidant activity of standard drug ascorbic acid shows maximum inhibition of 82.17% at 100µg/ml and ethanolic extract of synthesized silver nanoparticles shows the maximum inhibition at 100µg/ml is 67.33%. The antiarthritic activity of synthesized silver nanoparticles shows maximum inhibition of 72.07% in protein denaturation and for standard diclofenac sodium shows maximum inhibition of 81.98% at 100µg/ml. The HRBC method shows the maximum inhibition 68.10% for synthesized silver nanoparticles and 84.49% for standard diclofenac sodium at 100µg/ml. The antimicrobial activity was done against three bacterial and two fungal species which includes *Escherichia coli, Klebsiella pneumonia*, *Enterococcus faecalis, Staphylococcus aureus* and *Candida albicans, Candida vulgaris*. Among these species, *Escherichia coli* had a maximum zone of inhibition of 8mm at 100µg/ml concentration.

Keywords: Antioxidant, Antiarthritic and Antimicrobial activity, Synthesized silver Nanoparticles, *Annona squamosa*

INTRODUCTION

Nanotechnology is a branch of science that is very much popular due to its advanced development by using nanomaterials in suitable shapes and sizes in various fields like agriculture and medicine. The development in the field of nanotechnology remarkably consists of production and applications of nanoparticles in medicinal and pharmaceutical field **[1]**. Organic nanoparticles include Carbon nanoparticles, noble metal nanoparticles (silver and gold), semi-conductor nanoparticles (zinc oxide and titanium oxide) and inorganic nanoparticles are the magnetic nanoparticles. In medicine and pharma, targeted drug delivery is an important potential application of nanoparticles. Nanoparticles like Ag and Fe_3O_4 were used for efficient targeted drug delivery and selective destruction of tumor cell **[2-4]**. Nanoparticles have been successfully used in various medical applications such as cellular imaging **[5]** or in biosensors for proteins, DNA, carbohydrates, heavy metal ions **[6, 7]**, viruses **[8]**, and determination of blood glucose levels **[9]**. Silver nanoparticles (AgNPs) are majorly developing in many fields like medical, food, healthcare, industrial areas, consumer as they have unique chemical and physical features. Synthesis of nanoparticles is done using three different methods that are, physical, chemical and biological. In physical method, nanoparticles are done by evaporation-condensation process by using a tube furnace at atmospheric pressure **[10-13]**. In chemical methods, water or organic solvents are utilized in order to prepare the AgNPs **[14, 15]**. During the synthesis process of chemical method various toxic and hazardous by-products are released out. The disadvantage of using the chemical method is that they are highly expensive, low yield, toxic and they are harmful **[16]**. In contrary to the physical and chemical methods, biological methods that are done through AgNP has gained attention due to their capacity in reducing the effects of harmful by-products that are synthesized during the nanoparticle production **[17-20]**. The green synthesis method using the biological extracts has a wide attention due to the presence of vast metabolites that not only reduce the Ag^+ ions into AgNP but also prevents cluster formation and reduces toxicity **[21]**.

Rheumatoid arthritis (RA) is a chronic inflammatory, autoimmune disease that affects about 0.5-1% of the adult **[22]**. The epidemiology of RA has various aspects in the entire population **[23]**. Studies indicate that women are more likely to present RA

compared to men **[22]**. The prevalence of RA in women around menopause and pregnancy have led investigation to hypothesis that hormonal factors play a role in development of the disease **[24]**. Types of RA includes seropositive RA and seronegative RA. The other common type of arthritis is Juvenile RA. The strongest environmental risk factor associated with RA is cigarette smoking **[25]**. The cause of the chronic inflammation of RA is thin synovium to thick and the joint become puffy and swollen. Synovium invades and destroys the born and cartilage within joint **[26]**. Early diagnosis and treatment are important because it has a capacity to affect the ligaments, joints, tendons, muscles and cartilage **[27]**. Symptoms of RA includes pain in shoulder, stiffness, and pelvic girdle pain, mobility loose with fever, loss of body weight, fatigue, development of rheumatoid nodules under the skin and malaise **[28-30]**. Different strategies for treatment of RA are adopted to delay the disease progression and for the symptomatic relief **[31]**. Common medicines include non-steroidal anti-inflammatory drugs [NSAIDS], biologics [anti-TNF alpha], glucocorticoids and disease- modifying anti rheumatoid drugs (DMARDs) **[32]**. These therapies contain major drawbacks such as hypertension, cardiovascular disease, ulcer, neurodegenerative diseases and hepatotoxicity **[33]**.

Annona squamosa plant is also known as sugar apple or sweetsop and belongs to the family Annonaceae **[34]**. It is widely distributed in Brazil, Central America, Egypt, Peru and West Indies **[35]**. The chemical constituents of *Annona squamosa* consist of Coumarins, Tannins, Cardiac glycosides, saponins, flavonoids and carbohydrate **[36]**. Traditional uses of *Annona squamosa* include analgesic, anthelminthic, cancer, headache, digestive and carminative. The pharmacological activities of the *Annona squamosa* are antibacterial, anti-diabetic, antilipidemic, antioxidant, antimalarial **[37]**.

MATERIALS AND METHODS

Collection of plant material:

The roots of *Annona squamosa* were collected from Thanjavur district of Tamil Nadu.

Preparation of plant extract from root of *Annona squamosa*:

The root of *Annona squamosa* was washed with tap water to remove the contaminants. The roots is then dried and grinded into powdered form. The powder was separated from the mixture and stored in a container in a shady, dry and cool location for further use and research. To make extract, ethanol solution was used. 20 grams of powder sample was weighed and dissolved in 100ml of

ethanol solution for 24 hours. Hot percolation method was used for the extraction.

Preliminary phytochemical analysis and quantitative analysis of ethanolic extract of *Annona squamosa* root

The preliminary phytochemical qualitative analysis was done using standard procedures **[38]** and the quantification of phytochemical constituents such as flavonoids, tannins, saponins, alkaloids, phenol, and terpenoids (mg/g) present in *Annona squamosa* was found using standard procedure **[39]**.

Synthesis of Silver Nanoparticles

5ml of plant extract was dissolved in 50ml in silver nitrate ($AgNO_3$). When exposed to environment the Ag ions get reduced to Ag and a color change from a clear white to dark brown color is observed. UV spectral analysis was done in order to observe the synthesis of silver nitrate production **[40]**.

Characterization of Silver Nanoparticles

The characterization of silver nanoparticles occurs with the help of particle wavelength and functional group characteristics. By using the different techniques like, UV-visible spectra, FT-IR, SEM, XRD, EDAX, the morphology, crystalline nature size and silver nanoparticle composition is analyzed **[41]**.

UV -Visible Spectrum:

UV-VIS spectroscopy is an important technique that is used in the primary characterization of silver nanoparticles. The aqueous extract of root *Annona squamosa* was added to silver nitrate separately. The presence of $AgNO_3$ nanoparticle was confirmed by the color change from transparent white to reddish brown. The absorbance was set at 450-540nm in UV- vis spectrum after 24 hours of addition **[41]**.

FT-IR:

FTIR is abbreviated as Fourier Transform Infrared Spectroscopy which is also known as FTIR spectroscopy or FTIR analysis. FTIR detects the chemical properties like polymeric organic and inorganic materials and the synthesized $AgNO_3$ nanoparticles are scanned through infrared light.

FTIR absorbs at a range of 400-4000cm^{-1}. Absorption of various functional groups are observed at a particular frequency which gives to rise to further different characteristic absorptions **[41]**.

SEM:

The analysis of synthesized $AgNO_3$ nanoparticles were performed by Scanning Electron Microscope (SEM) in order to evaluate the surface morphology of nanoparticle. The synthesized AgNO3 Nanoparticles were prepared and kept for drying to remove the moisture content. The images were captured using FEI Quanta 250 SEM operating at 10 Kev **[41]**.

EDX Analysis:

The analysis of Energy Dispersive X-ray spectrometer proves the presence of Ag nanoparticles. The vertical axis shows the number of X- ray counts whereas, the horizontal axis shows energy in keV **[41]**.

X-ray Diffraction:

X-ray Diffraction (XRD)analysis is used to study the structural nanoparticles (range of 1-100nm). By the XRD analysis the positional value of the product (amorphous or crystallinity nature) can be observed. With respect to the d-spacing values the finger print regions of relative intensity are found in XRD analysis **[41]**.

Antioxidant activity using DPPH method

The silver nanoparticles synthesized from the roots of *Annona squamosa* were used for the antioxidant activity of stable DPPH enzyme by using ethanol as a solvent. Series of test tubes were taken in different types of concentrations ranging from 20-100µg/ml. Make up the 20,40,60,80 and 100µg/ml of sample into 1000ml by adding 980, 960, 940, 920 and 900 µg/ml of ethanol 1000µl of water is added to the negative control test tube. Add 500µl of DPPH enzyme after preparing the samples a different concentration. Absorbance is measured spectrophotometrically at 540nm after the addition of the enzyme. The percentage of inhibition is calculated by using following formula **[42]**.

Percentage (%) inhibition of DPPH activity = |(Control – Test)/control| × 100

Antioxidant activity using hydrogen peroxide method

20mM of hydrogen peroxide was added in phosphate buffer saline (PBS, pH 7.4). Various concentrations of the standard ascorbic acid (20-100 µg/ml) or root extract in ethanol were added to 2ml of hydrogen peroxide solution in PBS. The absorbance was measured at 230nm after 10mins **[43]**.

Percentage (%) inhibition of H_2O_2 activity = |(Control – Test)/control| × 100

Evaluation of anti-arthritic activity

Inhibition of protein denaturation model:

Control sample was prepared by using 1ml of egg albumin. At 20,40,60,80,100µg/ml of different concentrations, 2.8ml of phosphate buffer and 1000 g/ml distilled water was added to the test tube. Various concentration ranging from 20-100 µg/ml were made-up to 1000ml by adding ethanol at different concentrations like 980, 960, 940, 920, 900 µg/ml. The ethanolic extract of *Annona squamosa* roots at various concentrations (20,40,60,80,100 µg/ml) were added with 2.8ml of phosphate buffer solution and 1ml of egg albumin. 1 drop of concentrated hydrochloric acid is added to the test solution.

The test solution was incubated at 37ºC for 5 minutes. After incubation, the solution was analyzed in UV-Visible spectroscopy at 660nm **[44]**.

Percentage inhibition= [VC-V_t/V_c] x 100

Were,

V_t is absorbance of test sample

$V_c$is absorbance of control

Human red blood cell (HRBC) membrane Stabilization model

Preparation of reagents:

In order to prepare hypotonic solution,0.25g of sodium chloride was dissolved in 100ml of distilled water, whereas, to prepare isotonic solution0.45g of sodium chloride was dissolved in 100ml of distilled water. The pH of 1.89mg of disodium hydrogen phosphate is 7.4. To prepare phosphate buffer, 4mg of sodium chloride was dissolved in 100ml of distilled water. To prepare Alsever's solution, 1mg of dextrose, 6.4mg of sodium citrate and 0.025mg of NaCl was dissolved in 100ml of distilled water.

Preparation of suspension of human red blood cell (HRBC)

Blood sample from an arthritic patient was collected. The sterilized Alsever's solution is mixed with the blood sample and centrifuged at 2500rpm and the packed cells were separated and washed with isosaline solution after the centrifugation method.

Assay of membrane stabilizing activity:

The ethanol extract at various concentration like 20,40,60,80,100µg/ml are added to various concentrations of distilled water like 980, 960, 940, 920, 900 µg/ml. To prepare negative control, 1000µg/ml of distilled water, 1ml of phosphate buffer, 1ml of hypotonic saline and 100 µg/ml of HRBC in isotonic saline is added. 1ml of phosphate buffer, 1ml of hypotonic saline and various concentration of ethanol extract (20,40,60,80,100 µg/ml) are taken as test solution and 100 µg/ml of HRBC isotonic saline is added. The test solution was incubated at 37ºC for 5 minutes. After incubation, the solution is cooled. The absorbance of the solution is measured using UV-visible spectroscopy at 560nm.

Percentage protection = [VC-Vt /Vc] x 100

Were,

V_t is absorbance of test sample

V_c is absorbance of control

Antimicrobial activity of synthesized Silver Nanoparticle from the roots of *Annona squamosa*

Collection of test pathogens:

The Bacterial species *Escherichia coli, Klebsiella pneumonia, Enterococcus faecalis, Staphylococcus aureus* and the fungal species *Candida albicans*, *Candida vulgaris* are taken as samples are purchased from Microbial type culture and collection (MTCC), Chandigarh,

India and National chemical Laboratory (NCL), Pune, Maharashtra and India **[45]**.

Anti-bacterial activity by disc diffusion method

In 100 ml of distilled water was dissolved in Mueller-Hilton agar (MH agar of 3.8 g was prepared as media to grow the bacterial species. After media preparation, by using swap method, the culture added to the plate. Then at the top of the plate, the sterile paper discs with 6 mm diameter, which have soaked in different concentration (60,80and 100ul) of Ag nanoparticles along with positive (Amoxicillin) and Negative (Ethanol) controls was placed. The zone of Inhibition was measured after 24 hours in millimeter. The diameter of zone of Inhibition is directly proportional to the antibacterial activity **[45]**.

Anti-fungal Activity by disc diffusion Method

By using a swab method, the culture added in the plate. For the growth of fungal species, the Potato dextrose agar was prepared at a concentration of 2.9 g in 100 ml of distilled water. The sterile paper disc with 6 mm diameter which has soaked in different concentration (60,80 and 100ul) Ag Nanoparticles along with positive (fluconazole) and Negative (Ethanol) control was placed. At the top of the plate for the formation of zone of incubation, the plates are incubated for 24 hours, who's the diameter was measured in mm. The diameter of zone of incubation is directly proportional to the antibacterial activity **[45]**.

RESULTS AND DISCUSSION

Qualitative analysis of roots of *Annona squamosa*

The root of *Annona squamosa* showed the presence of terpenoids, flavonoids, saponins, tannins, alkaloids, steroids, glycosides, phlobatannins, protein, coumarins, emodin, anthraquinone, anthocyanin, carbohydrates, leucoanthocyanin, cardiac glycosides, xanthoprotein, phenol. The previous study by Vanitha Varadharajan *et al*., 2012 reported that the qualitative analysis of ethanolic extract of *Annona squamosa* leaves possessed the presence of alkaloids, carbohydrates, quinones, flavonoids, glycosides, triterpenoids, tannins, terpenoids, and anthocyanin **[38]**. Other study by Madhuri A. Theng and Dr. M. A. Channawar, 2022 stated that the ethanolic extraction by Soxhlet apparatus infers the presence of phytochemical compounds in *Annona squamosa* leaves are alkaloids [Mayer's and Wagner's test], coumarins [filter paper test], and steroids [Salkowaski reaction and Lieberman Burchard reaction] **[46]**.

Table 1: List of the phytochemical constituents present in ethanolic extract of *Annona squamosa* root

S.NO	PHYTOCHEMICAL CONSTITUENTS	RESULT
1.	Terpenoids	+++
2.	Flavonoids	+++
3.	Saponins	+++
4.	Tannins	+++
5.	Alkaloids	+++
6.	Steroids	+++
7.	Glycosides	++
8.	Phlobatannins	+
9.	Protein	++
10.	Coumarin	+++
11.	Emodin	+++
12.	Anthraquninone	+++
13.	Anthocyanin	+++
14.	Carbohydrate	+++
15.	Leucoanthoxyanin	+
16.	Cardiac glycosides	+++
17.	Xanthoprotein	+++
18.	Phenol	+++

+++ Stronglypresent; + Slightly present; ++ Moderatelypresent; - Absent

Figure 1: Qualitative analysis of *Annona squamosa* root

Quantitative analysis of roots of *Annona squamosa*

The quantitative analysis of the phytochemical constituents from the ethanolic extract of the root of *Annona squamosa* has flavanoids [0.011], tannins [0.019], saponins [0.015], alkaloids [0.013], phenol [0.012], and terpenoids [0.534]. The previous study by Mobasher MV *et al*., 2024 reported that the *Annona squamosa* fruit extract yielded total flavonoid content [16.75 ± .87mg QE/g DW] **[47]**. The level of saponin was [342 ± 3.97mg/g DW]. The total phenol content of *Annona squamosa* fruit extract was [25.48 ±2.56mg GAE/g DW]. In other research Anant Babu Marahatta *et al*., 2019 have been stated that the total phenolic compounds for hexane extracts of leaves and barks of *Annona squamosa* are calculated as 200.98 and 29.98mg/g GAE respectively. For methanol extract of leaves and barks of *Annona squamosa* are calculated as 217.82 and 60.48 mg/g GAE respectively. Total flavonoid contents of methanol extracts of leaves and barks are found to be 69.92 and 76.50 mg/g QE respectively **[48]**.

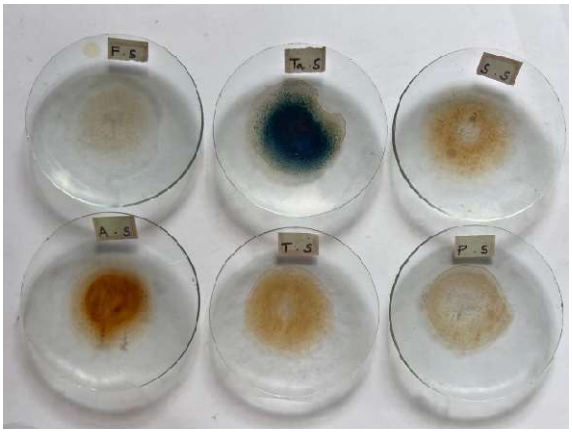
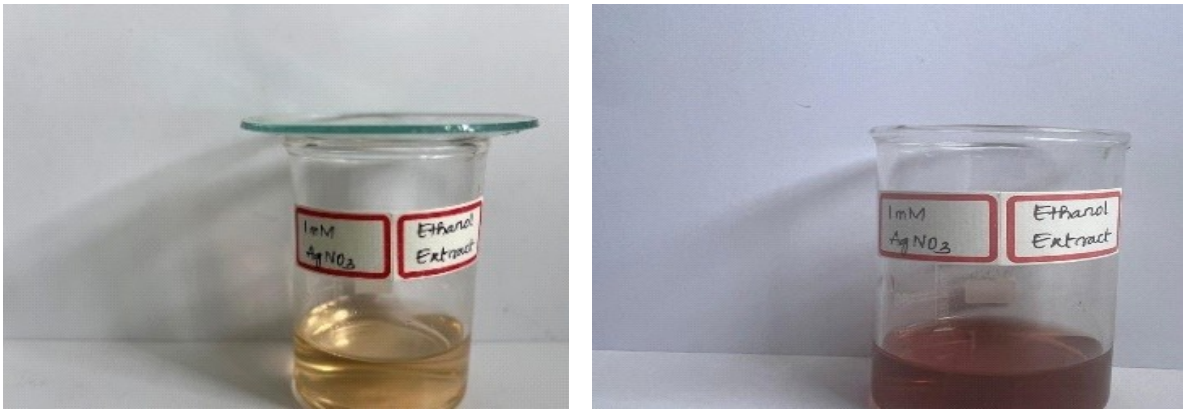


Figure 2: Quantitative analysis of *Annona squamosa* root

Synthesis of Silver Nanoparticles from *Annona squamosa*

The synthesis of silver nanoparticles is resulted by adding the ethanol extract from the plant sample containing $AgNO_3$ in the glass vial induces a color change. The initial transparent white color of the $AgNO_3$ transitioned into a reddish-brown color, as shown in (figure). The color transition reveals the presence of silver nanoparticles (AgNPs). The previous study by Maria Malik *et al*., 2022, reported that, the synthesis nanoparticles such as silver plays a major role in the plant which is employed as a reducing agent, whereby converting the metal ions into stable metal ions. The presence of AgNPs is

indicated either in golden brown or light brown color. The plant extracts were the primary source in reducing Ag+ ions to Ag particles. Hence, the color change has been examined and the color transition characteristics are used in the reduction mechanism and the synthesized size of AgNPs can be adjusted **[40]**. In another study by Ravindra B.K., and N.G. Patil., 2017, stated that the experiment using 50ml of silver nitrate solution and varying amount of leaf extract concentrations depicts a color change from colorless to dark brown color which indicates the reduction of silver nitrate to silver ions **[49]**.



BEFORE **AFTER**

Figure 3: Visual observation of synthesized silver nanoparticles

Characterization of Silver Nanoparticles

- **UV- Vis Spectroscopy:**

The color transition in the plant extract reveals the presence of silver nanoparticles (AgNPs). Further analysis in UV-vis spectroscopy proves the presence of plasma resonance band within the 455.9nm range and the shape of the nanoparticle is spherical. The previous study by Yogesh Kolekar *et al*., 2023, reported that the presence of high concentration of flavonoids and phenolic acids in *Annona squamosa* leaf extract determines that the silver nitrate can be bio-reduced to silver nanoparticles. The graphical representation of UV-Visible spectrophotometer depicts that there is a high peak and there is a formation of polydisperse nanoparticles **[41]**. In other research by Singaravelu Senthamilselvi *et al*., 2013 stated that the UV-Vis absorption spectra of *Annona squamosa* mediated biosynthesis of nanoparticles from leaf extract showed an absorption peak at 435 nm range **[50]**.

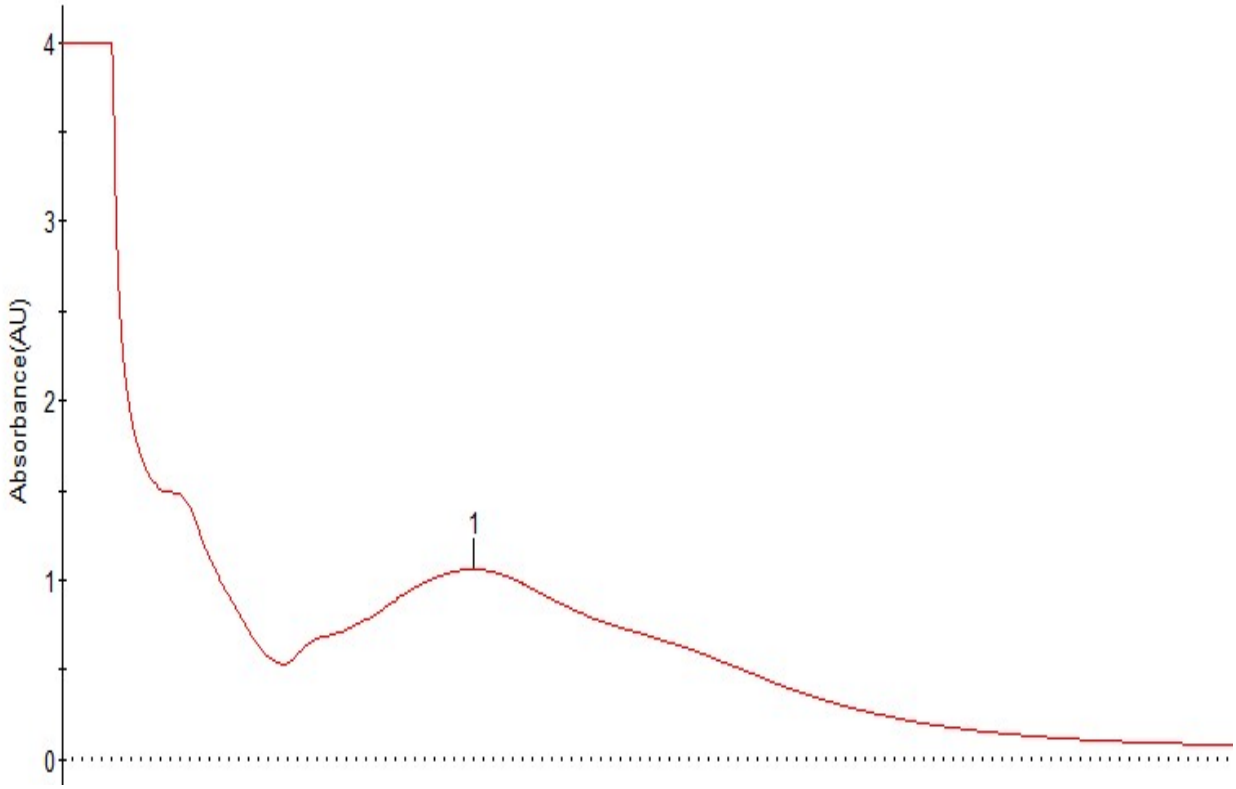


Figure 4: UV-Visiblespectrum of synthesized silver nanoparticles of sample

- **FT-IR**

FTIR is responsible for the reduction of silver ions in the functional group. The graphical representation of synthesized silver nanoparticles is illustrated in (Figure 5). The peaks that are highlighted in the graphical representation is 3451.18 cm^{-1},2074.95 cm^{-1},1637.73 cm^{-1},1033.16 cm^{-1} and 628.33 cm^{-1} which results in the presence of certain functional groups like phenolic or alcoholic group (OH), isothiocyanate(N=C=), alkene(-C=C-), ether(=C-O-C), alkyne(C-H).In the previous study by Yogesh Kolekar *et al*., 2023, reported that the peaks observed in the FTIR analysis were 614.57cm^{-1}, 729.63cm^{-1},830.06 cm^{-1},946.83 cm^{-1}, 1516.06 cm^{-1} and 3007.44 cm^{-1} which consists of alkyne, alkene and aromatic compounds. These functional groups interpret the C-H bend, C=C stretch and =C-H stretch. This study finally states the presence of functional groups in the synthesized silver nanoparticle **[41]**. In other research by Vimala Jose *et al*., 2021 stated that the prominent bands were observed at 096, 1232, 1319, 1386, 1636, 2208, 3400 cm^{-1}**[51]**.

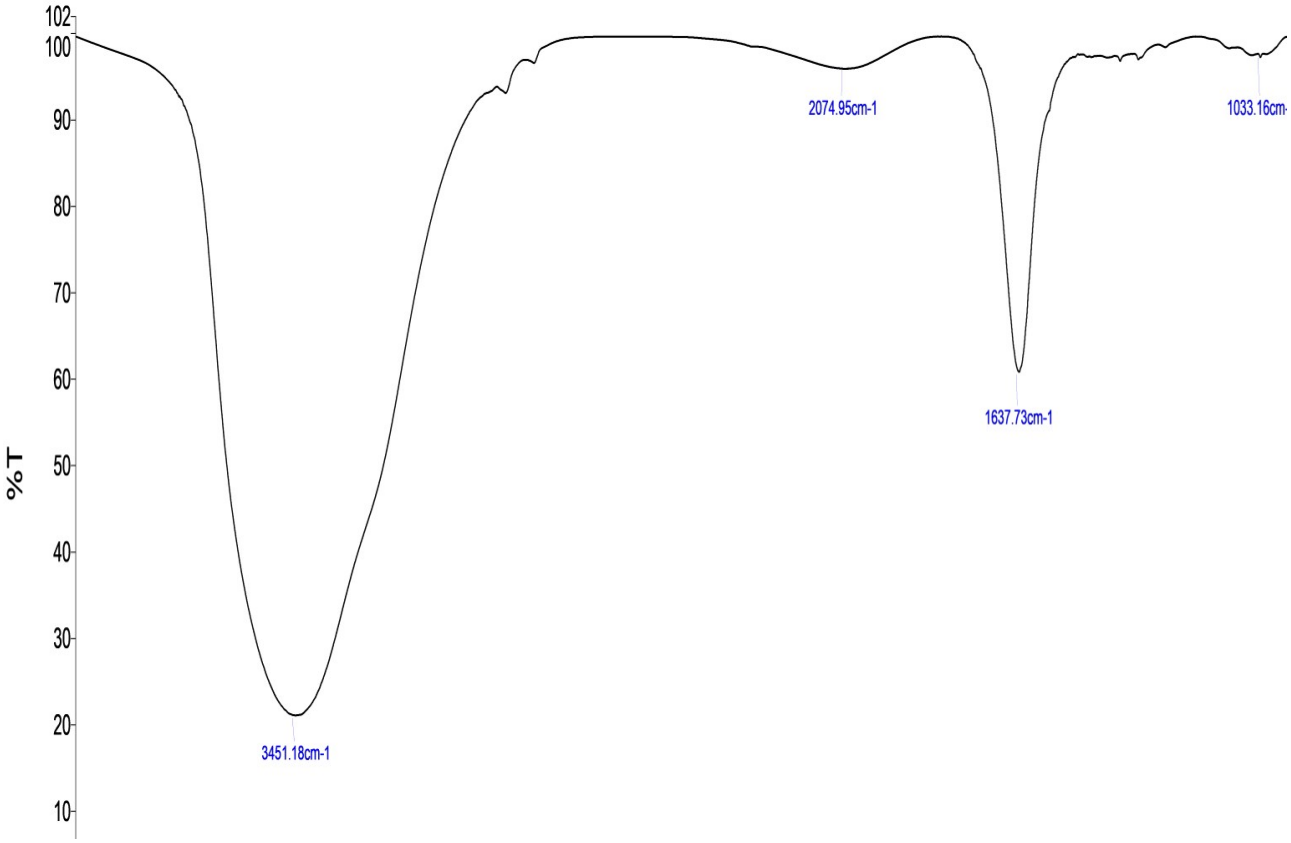


Figure 5: FT-IR spectra of synthesized silver nanoparticles

- **SEM**

The Scanning electron microscope (SEM) is responsible for the identification of particle size and the morphological structure of the synthesized silver nanoparticle. The diameter of the nanoparticles ranges from 74.08nm to 134.2nm hat has an average size of nanoparticles ranging approximately 200nm. The morphological structure of the nanoparticle is spherical shape and cubic shape. The previous study by Yogesh Kolekar *et al*., 2023, reported that morphology of the spherical-shaped silver particles is small and has bright tiny spots. The size of the synthesized silver nanoparticles ranges from 35-90nm and were magnified under 200nm scale **[41]**. In other research by Maria Malik stated that the analysis of *Annona squamosa* fruit extract mediated AgNPs exhibited range of 15 to 50nm and the *Annona squamosa* leaf extract mediated AgNPs revealed that the particle size of silver was in the range of 35 to 90 nm **[40]**.

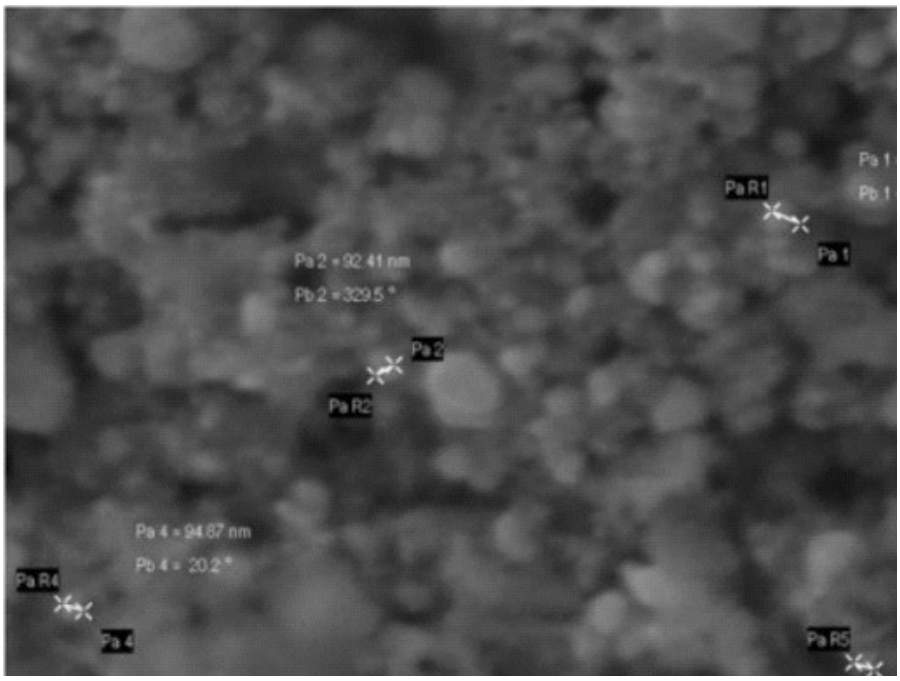


Figure 6: SEM image for synthesized silver nanoparticles

- **EDAX:**

Energy Dispersive X-ray (EDX) spectrometer is used to anticipate the presence of silver nanoparticles. The X-axis, exhibits the horizontal axis which indicates the energy in KeV, whereas the Y-axis, exhibits the vertical axis and displays the number of X-ray counts. (Figure 7) depicts the synthesized silver nanoparticles by EDAX analysis that demonstrates the additional peak of oxygen due to the attachment of biomolecules in the surface of the synthesized silver nanoparticles. So, the analysis of EDAX spectrum reports that the synthesized silver nanoparticles in the sample contains a weight percentage of 62.66%. The previous study by Vimala Jose *et al*.,2021 states that the analysis done for AgNPs present in *Annona squamosa* seed extract by EDAX exhibits a high-pitched absorption peak at 2.5KeV by confirming the presence of silver nanoparticles **[51]**. In another study R. Lakshmi Kalyani *et al*., 2019, the EDX spectra of *Annona squamosa* leaf extract-AgNPs were examined as 91.36% yield of elemental Ag L-line at voltage of 15 kv **[52]**.

Table 2: Elemental composition of synthesized silver nanoparticles

Element Line	Weight %	Weight % error	Atom %
Agcl K	37.34	± 0.59	56.63
Ag	62.66	± 0.67	43.37
Total	100.00		100.00

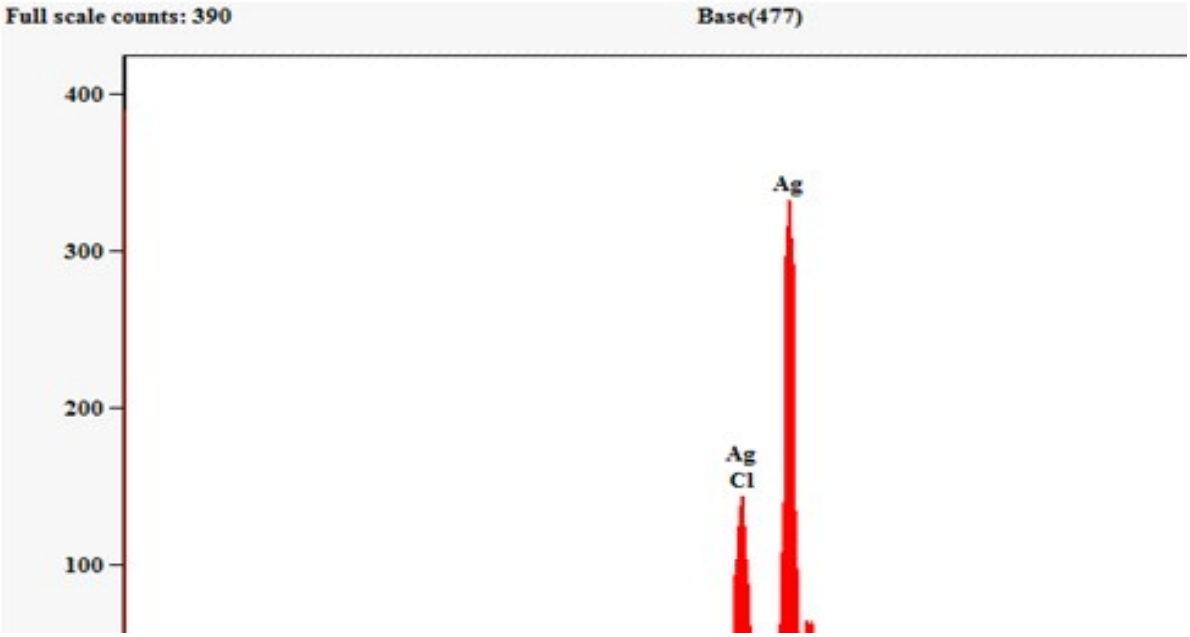


Figure 7: EDX spectrum of synthesized silver nanoparticles

- **X-ray diffraction (XRD):**

The X-ray crystallography explains about the crystalline nature of silver nanoparticles. The graphical representation of synthesized silver nanoparticles is depicted in (Table5, Figure 8). The strong diffraction peaks are observed at 2Θ values of 31.6941, 33.2129, 44.8823, 48.4125, 55.7269, and 69.9860, corresponding to the crystallographic facets (100), (002), (101), (102), (110), and (112) respectively. This pattern indicates a face-centered cubic structure for silver as per the JCPDS File No. 04-0783. Additionally, unassigned peaks are present in the graph, which could be attributed to the presence of phytochemicals in the extract that may be capping the surface of the nanoparticles. The previous study by Yogesh Kolekar *et al*., 2023, reported that, the production of AgNPs from *Annona squamosa* shows XRD pattern that has three different peaks with two values that ranges from 10-90. The crystalline nature of the synthesized silver nanoparticles was identified by the XRD band. The Bragg reflections of the (111) set of planes lattice were either demonstrated with the help of face-centered cubic constructions of silver were examined at two values 27.75º, 33.55º and 46.15º, 77.6º that were indexed as 110, 111, 121 and 311 **[41]**. In other research by Raju Vivek *et al*., 2012 stated that the spectra data revealed the occurrence of four major peak appeared with 2Θ values and these peaks are corresponding to the (111), (200), (222), (311), (222) planes of face-centered cubic phase which are closely in associated with standard card **[53]**.

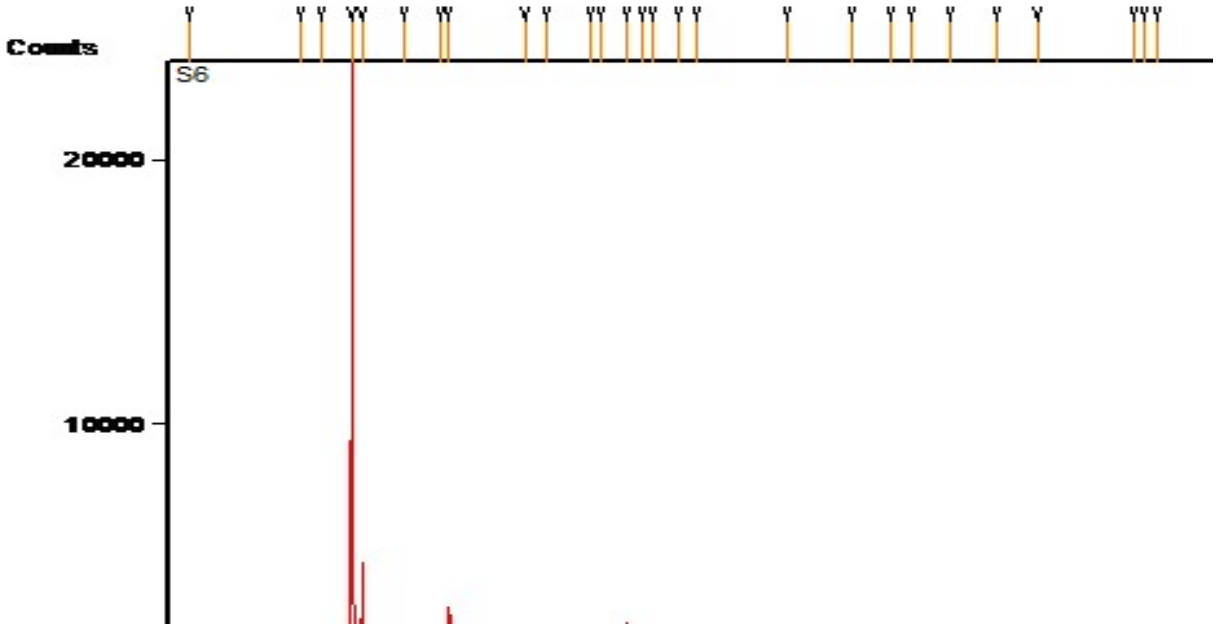


Figure 8: XRD patterns of silver nanoparticles synthesized using a sample

Antioxidant activity using DPPH method

The antioxidant activity reported a maximum inhibition by the DPPH method ranging from 35.29 to 61.76% at concentrations of 20-100µg/ml. Whereas, the maximum inhibition of standard drug ascorbic acid ranges from 46.07 to 78.43% at the same concentrations of 20-100µg/ml. The DPPH enzyme assay showed that the maximum inhibition ranges from 41.58 to 67.33 % at concentration of 20 to 100µg/ml. On the other hand, the standard drug ascorbic acid showed maximum inhibition ranges from 49.50 to 82.17 % at the concentration of 20 to 100 µg/ml. The previous study Abdalbasit Adam Mariad *et al*., 2012 reported that the antioxidant potential of *Annona squamosa* leaves, seedcake, root, and bark methanolic extract were demonstrated as IC_{50} value of 7.81,7.81,15.63,125.0 µg/ml **[42]**. In another study by Elumalai Nandhakumar and Parameswaran Indumathi, 2013, the DPPH activity reports that there is a color change in *Annona squamosa* leaf extract from purple to yellow color. The radical scavenging activity was determined with the methanolic extract IC_{50} value of 135.2 µg/ml, whereas, in contrast with aqueous extract depicts the IC50 value of 157.2 µg/ml **[54]**.

Table 3: Antioxidant activity of DPPH method

S. No.	CONCENTRATIONS (µg/ml)	SCAVENGING EFFECT (%)	
		Silver nanoparticles (AgNP) using root of *AnnonaSquamosa*	ASCORBIC ACID
1.	20	15.84	49.50
2.	40	25.74	54.46
3.	60	30.69	65.35
4.	80	38.61	73.27
5.	100	45.54	82.17

Figure 9: Antioxidant activity using silver nanoparticles from ethanolic extract of roots of *Annona squamosa* (DPPH)

Antioxidant activity using hydrogen peroxide method

The antioxidant activity reported a maximum inhibition by the hydrogen peroxide method ranging from 30.09 to 62.83% at concentrations of 20-100µg/ml. Whereas, the maximum inhibition of standard drug ascorbic acid ranges from 49.56 to 81.42% at the same concentrations of 20-100µg/ml. The previous study by Narasimharaju Kalidindi *et al*., 2015, stated that the hydrogen peroxide activity by in *Annona squamosa* leaf has maximum inhibition activity with IC_{50} value by using chloroform, aqueous and methanol extracts which resulted in 242.7µg/ml, 342.2 µg/ml and 453.7 µg/ml. Although, the ascorbic acid shows maximum inhibition activity with IC_{50} of 32.73 µg/ml **[43]**.

Table 4: Antioxidant activity of H_2O_2 method

S.NO	CONCENTRATIONS (µg/ml)	SCAVENGING EFFECT (%)	
		Silver nanoparticles (AgNP) using root of *Annona squamosa*	ASCORBIC ACID
1.	20	30.09	49.56
2.	40	37.19	53.98
3.	60	54.87	61.94
4.	80	60.18	66.37
5.	100	62.83	81.42

Figure 10: Antioxidant activity using AgNPs from ethanolic extract of roots of *Annona squamosa* (H_2O_2)

Evaluation of anti-arthritic activity

Inhibition of protein denaturation model

At different concentrations (20, 40, 60, 80, and 100 µg/ml), *Annona squamosa* has the ability to inhibit the protein denaturation like the control medication, diclofenac sodium. The diclofenac sodium medication expresses a higher effect of 81.98% at 100µg/ml, whereas the root extract presents a stronger effect of 72.07% at the same concentration expresses the inhibition of protein denaturation in anti-arthritic activity by using the diclofenac solution in *Annona squamosa* plant.

The previous study by Syed Zameer Ahmed Khader *et al*., 2022, reports the anti-arthritic activity of synthesized silver nanoparticles by the plant *Phoenix dactylifera* (Rothan dates). It shows maximum inhibition concentration as 68.44% at 500µg/ml concentration by Bovine Serum Albumin (BSA) assay **[55]**. In other research G.V.N Kiranmayi *et al*., 2021, showed that *Annona squamosa* leaves extract contains maximum inhibition of 71.42% at 200µg/ml for the evaluation of anti-arthritic activity using BSA method. From this, it can be clearly understood that the silver nanoparticles leaf extract of *Annona squamosa* has strong anti-arthritic activity **[44]**.

Table 5: Protein denaturation of roots of *Annona squamosa* by egg albumin method

S.NO	CONCENTRATION (µg/ml)	PROTEIN DENATURATION (%)	
		ROOTS OF *ANNONA SQUAMOSA*	DICLOFENAC SODIUM
1.	20	39.64	48.65
2.	40	50.45	54.95
3.	60	61.26	63.06
4.	80	65.77	70.27
5.	100	72.07	81.98

Figure 11: Protein denaturation of roots of *Annona squamosa*

Human red blood cell (HRBC) membrane stabilization model:

In-vitro examination for anti-arthritic activity was done by using the HBRC method. The control medication that was used Is diclofenac solution which stabilizes the HRBC membrane at different doses (20, 40, 60, 80, and 100 µg/ml). Even the *Annona squamosa* containing the ethanolic extract involves in stabilizing the HRBC membrane at different concentrations. In comparison to the standard diclofenac sodium that shows a stronger effect of 84.49% at 100 µg/ml, the root extract of *Annona squamosa* has a potent effect of 68.10% at 100 µg/ml. The HRBC assay has no discussions and has also not been reported in any of the previous studies, since this is the first report that is being studied about the HRBC assay in anti-arthritic activity.

Table 6: In vitro anti-arthritic activity of roots of *Annona squamosa* HRBC method

S. No.	CONCENTRATION (µg/ml)	HRBC ASSAY (%)	
		ROOTS OF *ANNONA SQUAMOSA*	DICLOFENAC SODIUM
1.	20	36.55	43.97
2.	40	46.55	56.03
3.	60	56.03	76.11
4.	80	62.07	78.45
5.	100	68.10	84.49

Figure 12: In vitro antiarthritic activity of roots of *Annona squamosa*

Antimicrobial activity in synthesized Silver Nanoparticles from *Annona squamosa* by disc diffusion assay method:

The anti-bacterial activity of synthesized silver nanoparticles by ethanolic extract from *Annona squamosa* study against microbes by disc diffusion method is expressed in **(Table 7)**. The leaf extract of *Annona squamosa* expresses certain growth inhibitory activity *Escherichia coli* (8mm), *Klebsiella pneumonia* (4mm), *Enterococcus faecalis* (5mm) and *Staphylococcus aureus* (6mm) at 100 µg/ml concentration. The fungi *Candida albicans* (5mm) and *Candida vulgaris* (4mm) had growth inhibition in the concentration 100µg/ml where it demonstrates the antimicrobial activity on all six microorganisms. Although, the extract exhibits a great inhibitory effort against pathogens. When the concentration of the

extract increases from 20-100 µg/ml, the inhibition system from the plant extract also increases. The anti-fungal and anti-bacterial activity identified from 41 traditional medicinal plant types that comes under 26 families by the ethanolic extraction method was done against 2 fungal and 3 bacterial species. They are *Candida albicans, Candida vulgaris* and *Escherichia coli, Klebsiella pneumonia*, *Enterococcus faecalis* and *Staphylococcus aureus* were tested in 41 plant species and out of 41, 39 plant species expressed antimicrobial activity against extra organism. The previous study by Anushman Bhattacharya *et al*., 2023 reported antimicrobial activity done in *Annona squamosa* seed which exhibited the zone of inhibition against gram positive *Staphylococcus aureus* bacteria and *Salmonella typhi* bacteria **[56]**. In another research R. Lakshmi Kalyani *et al*., stated that the anti-microbial activity in *Annona squamosa* leaf has a zone of inhibition in *Pseudomonas aeruginosa* which is 39.68mm and the zone of inhibition in *Staphylococcus aureus* is 18mm **[52]**.

Table 7: Antimicrobial activity of synthesized silver nanoparticle from *Annona squamosa*

	ZONE OF INHIBITION (mm)					
Concentration	**B1** ***(Escherichia coli)***	**B2** ***(Klebsiella Pneumonia)***	**B3** ***(Enterococcus faecalis)***	**B4** ***(Staphylococcus aureus)***	**F1** ***(Candida albicans)***	**F2** ***(Candida vulgaris)***
Standard	**12mm**	**6mm**	**8mm**	**7mm**	**6mm**	**9mm**
60 µg/ml	**5mm**	**2mm**	**3mm**	**2mm**	**2mm**	**3mm**
80 µg/ml	**7mm**	**3mm**	**4mm**	**3mm**	**3mm**	**4mm**
100 µg/ml	**8mm**	**4mm**	**5mm**	**6mm**	**4mm**	**6mm**
Ethanol	**0**	**0**	**0**	**0**	**0**	**0**

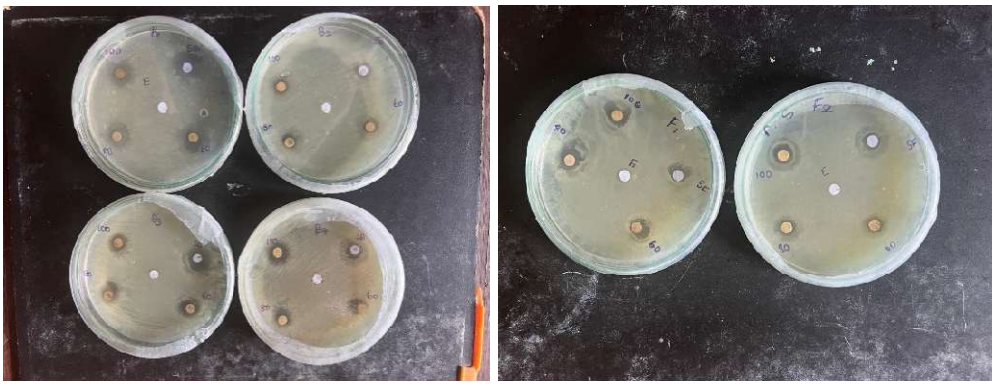


Figure 13: Antimicrobial activity of synthesized silver nanoparticle from *Annona squamosa*

CONCLUSION:

In the study, the root of *Annona squamosa* was extracted using the ethanolic extract and the silver nanoparticles were synthesized effectively by using the green method. The test for phytochemical screening of

synthesized silver nanoparticles extracted using ethanol of root of *Annona squamosa* resulted the presence of phytochemical constituents that includes terpenoids, flavonoids, saponins, tannins, alkaloids, steroids, glycosides, phlobatannins, proteins, coumarins, emodin's, anthraquinone, anthocyanin, carbohydrates, leucoanthocyanin, cardiac glycosides, xanthoproteins, and phenol. Whereas, the quantitative analysis showed the presence of secondary metabolites such as flavonoids, tannins, saponins, alkaloids, phenol, and terpenoids. The peak intensity of synthesized nanoparticles was characterized using UV-Visible spectroscopy, observed at 455.9nm. FTIR resulted the functional group such as phenolic or alcoholic group (OH), isothiocyanate(N=C=), alkene(-C=C-), ether(=C-O-C), alkyne(C-H). The scanning electron microscope analyzed the synthesized silver nanoparticles were spherical in shape. The XRD analysis was carried out to determine the crystalline nature of the silver nanoparticles and the diffraction peaks were observed at 2Θ values of 31.6941, 33.2129, 44.8823, 48.4125, 55.7269, and 69.9860. The analysis of EDAX spectrum reported that the synthesized silver nanoparticles in the sample contains a weight percentage of 62.66%. By performing antioxidant activity of synthesized silver nanoparticles using *Annona squamosa* root showed the maximum inhibition ranges from 41.58 to 67.33 % at concentration of 20 to 100µg/ml for DPPH method, whereas, the H_2O_2 method showed the maximum inhibition ranges from 30.09 to 62.83% at concentration of 20-100µg/ml. The antiarthritic activity for protein denaturation method of synthesized silver nanoparticles showed the inhibition 39.64 to 72.07% at concentration of 20-100µg/ml and the HRBC method of antiarthritic activity showed 36.55 to 68.10% of synthesized silver nanoparticles using ethanolic extract of root of *Annona squamosa.* Also, antimicrobial activity through disc diffusion method of silver nanoparticles was exhibited. From that Escherichia coli showed maximum zone of inhibition of 8mm radius at a concentration of 100 µg/ml.

CONFLICTS OF INTERESTS

The author's declaration that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

AUTHOR`S CONTRIBUTION

All authors have equally contributed to this manuscript.

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